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PATHOGENESIS AND IMMUNITY

Nectin-4 Interactions Govern Measles Virus Virulence in a New Model of Pathogenesis, the Squirrel Monkey (*Saimiri sciureus*)

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ABSTRACT In addition to humans, only certain nonhuman primates are naturally susceptible to measles virus (MeV) infection. Disease severity is species dependent, ranging from mild to moderate for macaques to severe and even lethal for certain New World monkey species. To investigate if squirrel monkeys (*Saimiri sciureus*), which are reported to develop a course of disease similar to humans, may be better suited than macaques for the identification of virulence determinants or the evaluation of therapeutics, we infected them with a green fluorescent protein-expressing MeV. Compared to cynomolgus macaques (*Macaca fascicularis*) infected with the same virus, the squirrel monkeys developed more-severe immunosuppression, higher viral load, and a broader range of clinical signs typical for measles. In contrast, infection with an MeV unable to interact with the epithelial receptor nectin-4, while causing immunosuppression, resulted in only a mild and transient rash and a short-lived elevation of the body temperature. Similar titers of the wild-type and nectin-4-blind MeV were detected in peripheral blood mononuclear cells and lymph node homogenates, but only the wild-type virus was found in tracheal lavage fluids and urine. Thus, our study demonstrates the importance of MeV interactions with nectin-4 for clinical disease in the new and better-performing *S. sciureus* model of measles pathogenesis.

IMPORTANCE The characterization of mechanisms underlying measles virus clinical disease has been hampered by the lack of an animal model that reproduces the course of disease seen in human patients. Here, we report that infection of squirrel monkeys (*Saimiri sciureus*) fulfills these requirements. Comparative infection with wild-type and epithelial cell receptor-blind viruses demonstrated the importance of epithelial cell infection for clinical disease, highlighting the spread to epithelia as an attractive target for therapeutic strategies.

KEYWORDS cellular receptors, immune suppression, measles virus, pathogenesis, primate models

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Despite the availability of a safe and effective vaccine and the successes of the ongoing measles virus (MeV) eradication campaign (1), the virus remains one of the most important vaccine-preventable childhood infections. Because of its highly contagious properties, protective immunity in >95% of a population is required to prevent MeV outbreaks (2, 3). This target is not only difficult to reach but even harder to maintain, as recurrent outbreaks in North America and Europe illustrate. Strategies that seek to interrupt the chain of transmission would thus complement the vaccination efforts and could accelerate the eradication endgame.

Only humans and certain nonhuman primate species are natural hosts for MeV. Among susceptible primates, certain New World species are highly sensitive and develop a severe and sometimes even lethal disease (4–6), while macaques tend to be slightly less affected than most human patients. Nonetheless, macaques are the most frequently used animal model, in part due to the availability of reagents to assess immune responses and to identify target cells.

In recent years, several important insights into the early stages of MeV pathogenesis have been gained from such studies in rhesus or cynomolgus macaques (7–15). After inoculation via aerosol, which most closely reproduces natural infection, virus is first detected in immune cells of the lower respiratory tract (11). These cells may transport the infection to the draining lymph nodes, where it spreads to immune cells expressing the signaling lymphocytic activation molecule (CD150/SLAM) and then disseminates to other lymphatic tissues and organs (8, 12).

Spread to and within epithelia requires interaction with nectin-4 (14, 16, 17). This protein is expressed at high levels in the trachea, which acts as a site of virus release. Even though an MeV unable to interact with nectin-4 is not shed into the airways of infected macaques, its virulence did not differ from that of the wild-type strain, since a mild rash and short-lasting anorexia were the only clinical signs (13). An analogously constructed nectin-4-blind canine distemper virus (CDV), a related morbillivirus that causes lethal disease in ferrets, did spread efficiently throughout the immune system and caused severe immunosuppression but no infection of epithelial tissues and no clinical disease (18).

To investigate the contribution of epithelial infection to clinical disease in MeV in more detail, we infected squirrel monkeys (*Saimiri sciureus*) with either a nectin-4-blind or a wild-type MeV and compared the infection levels in immune and epithelial tissues. These viruses expressed the enhanced green fluorescent protein (eGFP) from an additional transcription unit inserted in their genome, which facilitated visualization of the infection in monkeys. The fact that the same virus stocks were used in a previous study in cynomolgus macaques (10) allowed side-by-side comparison of the pathogenesis in the two species. Squirrel monkeys were sacrificed on days 7 and 12, reflecting the peak of immune cell and epithelial infections, respectively, in macaques (7). Blood and tracheal lavage samples were collected, and tissue samples were harvested to assess the impact of abolished nectin-4 binding on viral load, severity of immunosuppression, and dissemination in the different tissues.

RESULTS

Squirrel monkeys develop more severe clinical disease than cynomolgus macaques after wild-type MeV infection. While there are reports of MeV causing more severe disease in different New World primate species, including squirrel monkeys, than in macaques (4, 19), it is unclear if this effect is strain or species dependent. Here, we infected squirrel monkeys with the recombinant wild-type strain IC323, which expresses the eGFP gene from an additional transcription unit inserted upstream of the nucleocapsid (N) gene (20). Pathogenesis of this virus has been extensively characterized in rhesus and cynomolgus macaques, where only minimal clinical signs and mild and transient immunosuppression were monitored (10). To ensure a fair side-by-side comparison of MeV pathogenesis in different species, we infected the squirrel monkeys with the same dose of virus from the same batch that we previously used to infect cynomolgus macaques (10).

All squirrel monkeys developed a severe generalized rash (Table 1), and all animals experienced at least three out of the four clinical signs fever, diarrhea, rhinorrhea, and sneezing or congestion, while the only clinical sign in macaques was a mild localized rash (Table 1). The eGFP expression associated with MeV infection allowed us to easily document the extent of viral spread in different tissues. Differences were noted in the severity of rash, with only a few low-fluorescing spots in macaques and widespread bright foci in squirrel monkeys (Fig. 1). In the latter, there also was widespread infection in slices of fresh lymph nodes and other immune tissues (Fig. 1), and multifocal infection in

TABLE 1 Clinical signs of MeV and MeV-N4^{blind} infection observed in cynomolgus macaques and squirrel monkeys

	No. with sign/total no. for primate with virus		
		Squirrel monkey with:	
Clinical sign	Cynomolgus macaque with MeV	MeV	MeV-N4^{blind}
Rash	5/5 (weak)	8/8 (severe)	6/6 (weak)
Fever	0/5	7/8	1/6
Diarrhea	0/5	4/5	0/3
Rhinorrhea	0/5	4/5	0/3
Sneezing or congestion	0/5	3/5	0/3

epithelial organs (Fig. 1), whereas the infection in the corresponding macaque tissues was less pronounced and more localized (Fig. 1). These observations were confirmed by immunohistochemistry staining of paraffin-embedded lymph node and lung sections, where the level of infection in macaque tissues (Fig. 2A) was significantly lower than in the corresponding squirrel monkey tissues (Fig. 2B, left, and C).

Cell-associated titers in squirrel monkeys reached up to 10^4 infectious doses per 10^6 peripheral blood mononuclear cells (PBMCs) on day 7, when titers in macaques were just above the detection threshold of 1 (Fig. 3A, compare solid triangles and circles). Even at the peak of infection on day 9, viral load in macaques did not exceed 10^3 infectious doses per 10^6 PBMCs (see reference 10). On day 12, the viral load in both species was declining, with several animals having cleared the virus and the positive animals showing titers below 10^3 (Fig. 3A). While the initial drops in white blood cell count were similar in the two species, the overall loss was more dramatic in squirrel monkeys (Fig. 3B, compare solid triangles and circles). A similar tendency was also observed for the inhibition of nonspecific proliferation activity (Fig. 3C, compare solid triangles and circles), where the extent of inhibition was significantly more severe in squirrel monkeys. Thus, this species is indeed more sensitive to MeV-induced clinical disease and immunosuppression than macaques.

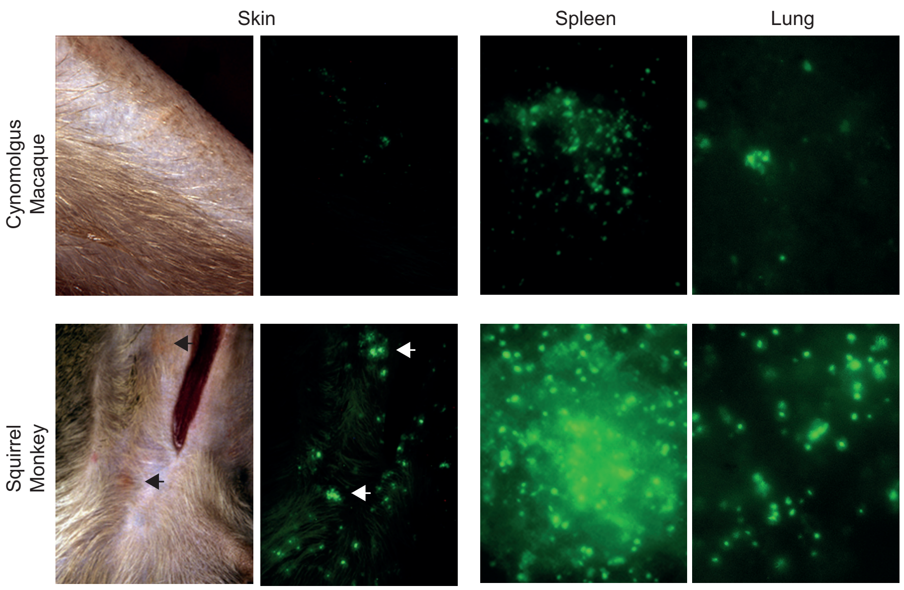


FIG 1 Infection levels associated with wild-type MeV infection in macaques and squirrel monkeys. Groups of 5 female macaques and 8 female squirrel monkeys were inoculated intranasally with $10^{4.5}$ $TCID_{50}$ of wild-type MeV. Photographs of skin rash and fluorescent signals in skin and tissues in animals sacrificed on day 12. Photographs and fluorescent images of the skin were taken during necropsy. The red wedge is the necropsy cut line, and arrows indicate areas of intense rash. Fluorescent images of fresh spleen and lung tissue were photographed using an inverted fluorescence microscope at 100-fold magnification.

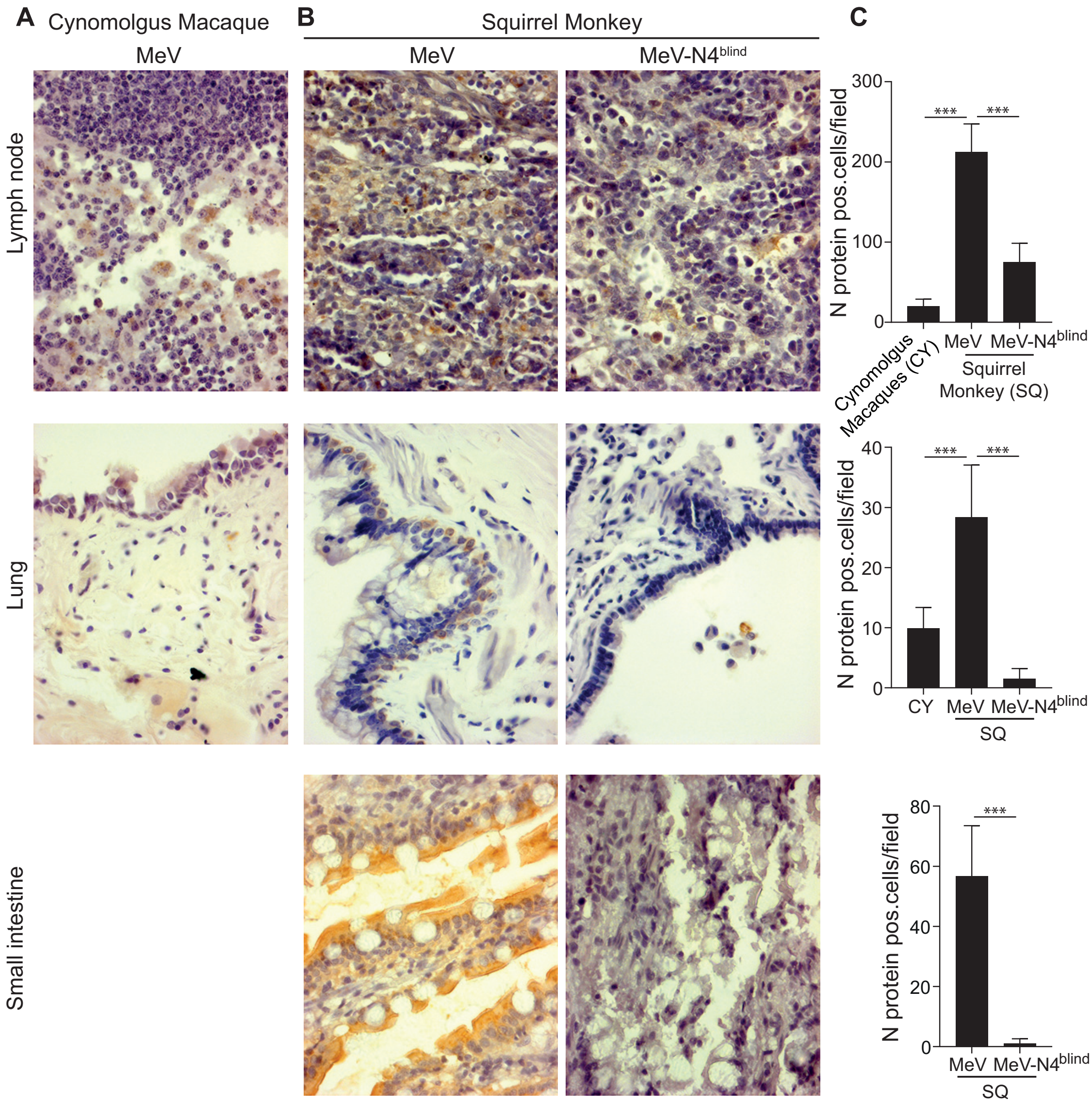


FIG 2 Wild-type and nectin-4-blind MeV infection levels in macaque and squirrel monkey tissues. (A and B) Paraffin-embedded sections from wild-type-MeV-infected macaques (A), wild-type-MeV-infected squirrel monkeys (B, left panels), and MeV-N4^{blind}-infected squirrel monkeys (B, right panels) harvested on day 12 after infection were stained with a nucleoprotein-specific antibody revealed with the brown chromogen DAB and counterstained with hematoxylin. Slides were photographed at ×400 magnification. (C) Quantification of MeV N protein-positive cells per visual field. For each group, MeV N protein-positive cells were counted in three separate visual fields on slides from the three animals sacrificed at that time point. The columns show the group means, the error bars indicate the standard deviations, and asterisks describe levels of statistical significance (***, $P < 0.001$).

Nectin-4 is essential for MeV clinical disease but not immunosuppression. In addition to infection of a group of eight squirrel monkeys with the wild-type eGFP-expressing MeV, a group of six animals was inoculated with its nectin-4-blind derivative, MeV-N4^{blind} (13). Again, this group was infected with the same dose of virus from the same batch used in a previous cynomolgus macaque study (10). In contrast to the wild-type virus, MeV-N4^{blind} did not cause any clinical signs apart from a mild rash (Fig. 4 and Table 1), and only one animal developed a short-lived fever (Table 1). There was no difference in viral loads in PBMCs, and the average titer on day 7 was slightly, but not significantly, higher in the MeV-N4^{blind}-infected group (Fig. 3A, compare solid and empty circles). White blood cell counts at days 7 and 12 postinfection (Fig. 3B, compare solid and empty circles) and lymphocyte proliferation activities at the same time points (Fig. 3C, compare solid and empty circles) were similar in monkey groups infected with the two viruses.

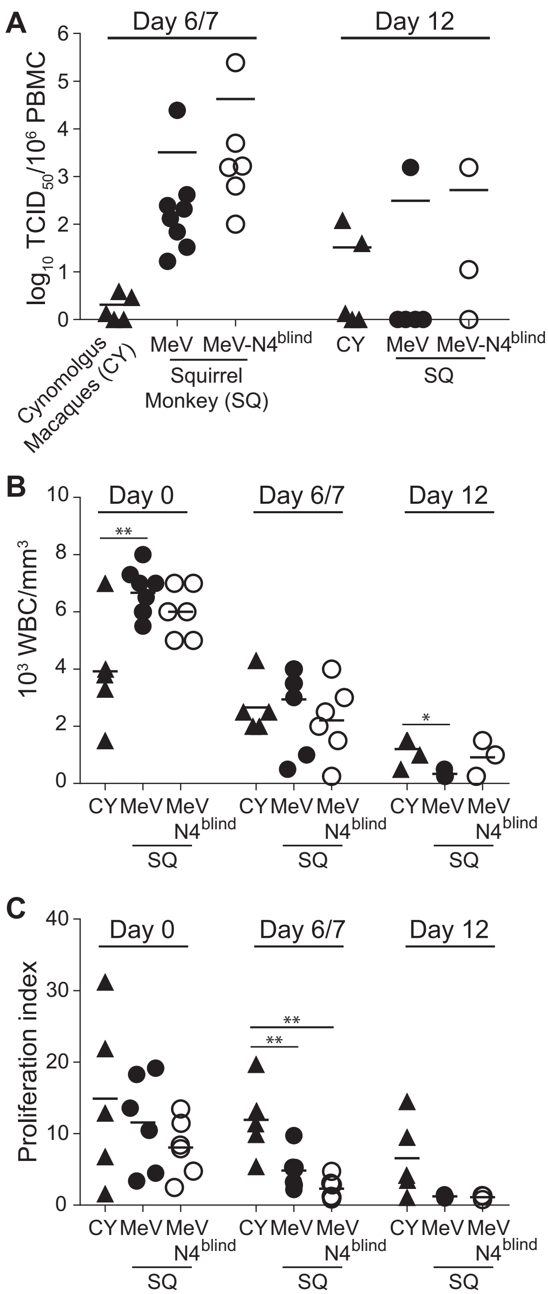


FIG 3 Cell-associated viremia and immunosuppression levels in wild-type-MeV-infected macaques and squirrel monkeys infected with wild-type MeV or MeV-N4[blind]. Groups of 8 and 6 female squirrel monkeys were inoculated intranasally with $10^{4.5}$ $TCID_{50}$ of wild-type MeV or MeV-N4[blind], respectively. Data from a previously published study (10) with a group of 5 female cynomolgus macaques inoculated intranasally with $10^{4.5}$ $TCID_{50}$ of the same stock of wild-type MeV are also shown. Levels of cell-associated viremia (A), white blood cell (WBC) counts (B), and *in vitro* lymphocyte proliferation activity levels (C) at the indicated days postinfection. Cell-associated viremia is expressed as $\log_{10}$ $TCID_{50}$ per 10^6 PBMCs, white blood cell count is expressed as 10^3 cells per mm^3, and proliferation index reflects the ratio of BrdU incorporation in PHA-stimulated and nonstimulated PBMCs. Each symbol represents one animal, thick horizontal black lines indicate the group means, and asterisks describe levels of statistical significance (*, $P < 0.05$; **, $P < 0.01$).

On day 7 after infection, when the virus loads reached average values of around 10^2 to 10^3 infectious doses per 10^6 PBMCs (Fig. 3A), animals in the two groups had similar titers in lymph node homogenates (Fig. 5A) and showed similar infection levels in tissue sections (data not shown). Even though virus could be isolated from only one of the animals in the wild-type-MeV-infected group on day 12 (Fig. 5A), the number of MeV

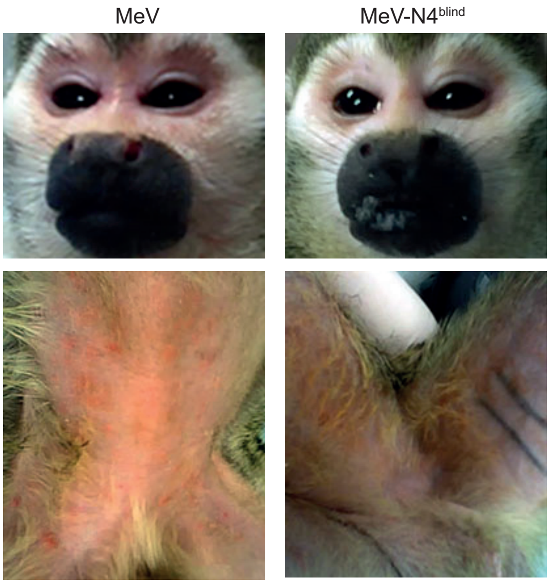


FIG 4 Rash in squirrel monkeys infected with wild-type MeV or MeV-N4blind. Photographs of animals 12 days after infection with wild-type MeV or MeV-N4blind.

N protein-positive cells per visual field in slides from wild-type-MeV-infected animals was significantly higher than in the group infected with the nectin-4-blind virus (Fig. 2C, top). Virus was detected in tracheal lavage fluid of both groups on day 7 (Fig. 5B), but only the wild-type MeV group shed virus in the tracheal lavage fluid (Fig. 5B) or urine (Fig. 5C) at days 12 and 21, respectively, after virus spread to epithelial tissues. This suggests that the virus infectivity documented on day 7 may be of immune cell origin and perhaps related to the microinjuries during the collection process. No virus was isolated from the cerebrospinal fluid (CSF) of any animal (data not shown).

The lack of epithelial cell infection in the group infected with MeV-N4blind was also reflected in the lack of MeV N protein-positive cells in sections of lung and small intestine harvested on day 12 (Fig. 2B, top panels). While there was widespread staining in lung epithelial cells of wild-type-infected animals, usually associated with areas of hyperplasia and desquamation (Fig. 2B, left middle panel, and C, middle graph), the only few positive cells found in sections from animals infected with MeV-N4blind had a monocytic phenotype (Fig. 2B, right middle panel, and C, middle graph). This difference was even more striking in gastrointestinal tissue sections (Fig. 2B, bottom panels, and C, bottom graph). Thus, as previously observed in macaques, MeV-N4blind reached infection levels similar to those of the wild-type virus in immune tissues at early disease stages but was unable to spread to epithelial cells.

Efficiency of *in vitro* PBMC infection recapitulates species susceptibility to MeV infection. To determine if the efficiency of infection and replication in PBMCs correlates with disease severity in the respective species, we compared the *in vitro* replications of MeV and MeV-N4blind in purified human, macaque, and squirrel monkey PBMCs. Both viruses replicated efficiently in phytohemagglutinin (PHA)-activated human PBMCs, reaching infection levels of 30 to 40% within 24 h (Fig. 6A). Infection levels of 10 to 15% were observed in squirrel monkey PBMCs, whereas only around 5% of macaque PBMCs became infected (Fig. 6A), irrespective of the virus used. The susceptibility of squirrel monkey PBMCs to MeV infection is thus intermediate between that of human and macaque PBMCs. We aligned the H protein-binding domains, consisting of residues 51 to 70, of SLAM proteins from humans, squirrel monkeys, and macaques (Fig. 6B). Residues 58 to 61 and 63 have previously been shown to be important for attachment by the H protein (21, 22). The squirrel monkey SLAM protein differed by an isoleucine at position 63 compared to valine in human and macaque SLAM, but this difference has

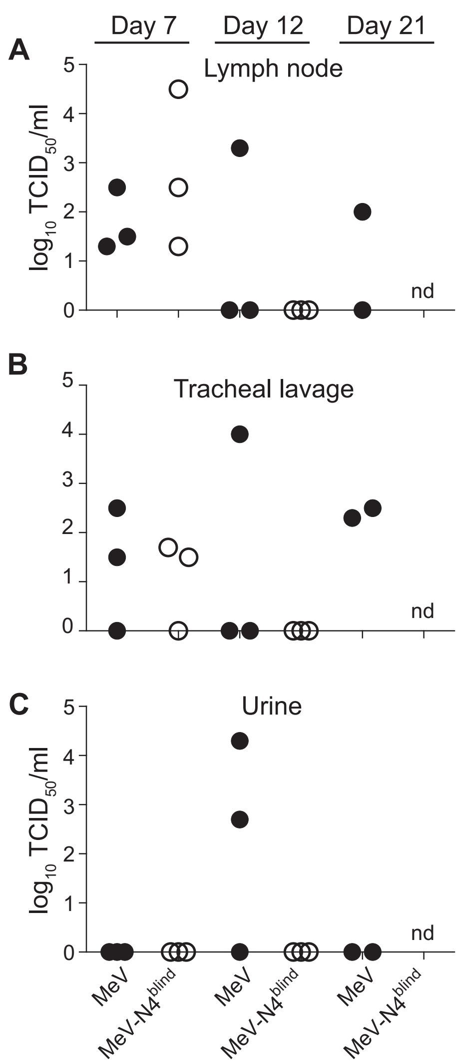


FIG 5 Infection levels in lymph nodes and body fluids. Virus titers were quantified in mesenteric lymph nodes (A), tracheal lavage fluids (B), and urine (C) from animals sacrificed at different times after inoculation as indicated above the panels. Three animals of each group were sacrificed on days 7 and 12, and two animals infected with wild-type MeV were sacrificed on day 21. Titers were determined by limited dilution and are expressed as $\log_{10}$ $TCID_{50}$/milligram of tissue or milliliter, respectively. nd, not done.

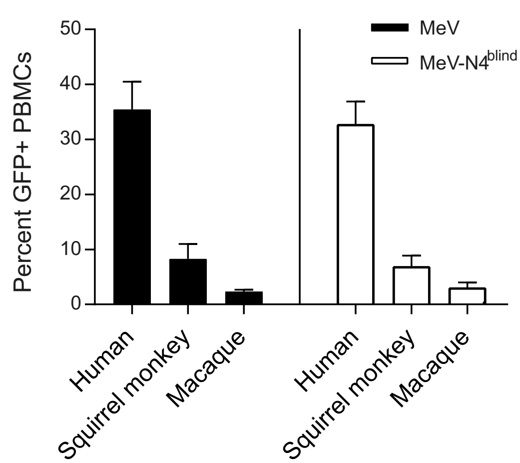
not been associated with a reduced capacity to bind the H protein. The observed differences in MeV replication in the PBMCs of the respective species may thus reflect the influence of postentry restriction factors rather than H protein-binding capacity.

DISCUSSION

While great progress has been made over the last several years in understanding MeV immunopathogenesis using the macaque model, characterization of MeV clinical disease has largely relied on human case reports due to the lack of clinical signs in macaques. Here, we demonstrate that squirrel monkeys are good models of measles pathogenesis. First, disease is more severe in this species than in cynomolgus macaques infected with the same wild-type MeV strain. Second, we document the critical contribution of nectin-4 interactions to clinical disease.

Squirrel monkeys are the New World primate species most frequently used in biomedical research (23). They are relatively easy to handle and maintain under laboratory conditions and naturally develop several cardiovascular and neurologic conditions seen in human patients (24–26). In addition, they are susceptible to a

A



B

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                      *** ** *
Human          51 RINKSMNKSI HIVVTMAKSL 70
Squirrel monkey 51 ...T...... ...I...... 70
Macaque        51 .......... .......... 70
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FIG 6 Susceptibility of human and nonhuman primate PBMCs to *in vitro* MeV infection. (A) PBMCs were isolated from humans, pig-tailed macaques, and squirrel monkeys and were infected with either wild-type MeV or MeV-N4^{blind}. Cells were harvested 24 h after infection and fixed in 2% PFA. The percentage of GFP-positive lymphocytes was then quantified by fluorescence-activated cell sorting. (B) SLAM protein alignments from humans, squirrel monkeys, and macaques. Residues 58, 59, 60, 61, and 63 (indicated by asterisks) are known to be directly involved in H protein binding. Accession numbers: human (*Homo sapiens*), NM_003037; squirrel monkey (*Saimiri boliviensis*), XM_003937956; macaque (*Macaca fascicularis*), XM_015454807.

number of human pathogens (23), including yellow fever (27), influenza (28), Nipah (29), and measles (4) viruses. Squirrel monkeys have been used to study the toxicity of oncolytic MeVs, and treated animals do not experience adverse side effects or pathology from these vaccine-strain viruses (30). While MeV infections in macaques are generally characterized by high morbidity but mild disease (7), outbreaks in New World species lead to severe clinical disease and can even result in mortality (5). By comparing the pathogeneses of the same viruses in macaques and squirrel monkeys, we show that the latter are indeed more sensitive to infection. Only squirrel monkeys closely reproduced the clinical disease spectrum seen in MeV-infected children, including the characteristic rash, fever, and respiratory and gastrointestinal signs. Thus, this species is a promising model to investigate the underlying mechanisms.

The contribution of nectin-4 interactions to morbillivirus pathogenesis has been previously investigated in macaques and ferrets infected with nectin-4-blind derivatives of MeV and CDV, respectively (13, 14, 18). In ferrets, the wild-type and nectin-4-blind viruses initially caused the same extent of infection in immune tissues and resulted in similar levels of immunosuppression (18). In contrast to the wild-type virus, which went on to cause severe clinical disease leading to death in all infected animals, ferrets infected with the nectin-4-blind virus did not develop any clinical signs and gradually overcame the immunosuppression, demonstrating the importance of epithelial infection for clinical disease in this model (18). In macaques, the viral loads in PBMCs and the extents of immunosuppression were also similar for the two viruses, and the nectin-4-blind derivative was unable to infect epithelial cells. However, the impact on clinical disease remained unclear due to the overall mild course (10, 13). The comparison of the two viruses in squirrel monkeys demonstrates that the causal link between nectin-4-mediated epithelial cell infection and clinical disease is shared by MeV and CDV and most likely all morbilliviruses. Thus, strategies that interrupt spread to epithelial cells would not only prevent disease in infected individuals but might also reduce transmission and could thus contribute to the success of the MeV eradication campaign.

While humans are the only natural host for MeV, other primate species can be infected, and indeed some populations even show serological evidence of exposure (31, 32). There is no evidence that MeV circulates actively in nonhuman primate species, whose population sizes may be too small to sustain the MeV transmission cycle (33). We show here that macaque and squirrel monkey PBMCs are less susceptible to *in vitro* MeV infection than human PBMCs. MeV H interacts with SLAM across an extended binding surface consisting of four sites (34), and mutations in many of these SLAM residues abolish or greatly reduce H protein binding. This interaction is a primary barrier to infection by morbilliviruses, particularly for CDV and cetacean morbillivirus (CeMV) infection of various terrestrial and marine carnivores (22, 35–37). However, our analysis of the H protein-binding domain of the human, squirrel monkey, and macaque SLAM proteins did not show any amino acid differences associated with altered binding properties. This implies that host-specific postentry factors determine efficient MeV infection and spread in different primate species and may thus prevent its circulation in wild populations.

MATERIALS AND METHODS

Cells and viruses. Vero/human SLAM (hSLAM) cells (38) were kindly provided by Y. Yanagi (Kyushu University, Fukuoka, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 5% fetal bovine serum (FBS; Invitrogen) and 0.5 mg/ml of G418 (Invitrogen). The eGFP-expressing MeV wild-type strain IC323 (20, 39) and its nectin-4-blind derivative MeV-N4^{blind} (13, 14) were propagated in Vero/hSLAM cells. Virus titers were quantified by limited dilution method and expressed as 50% tissue culture infectious dose ($TCID_{50}$).

Animal experiments. The experimental setup of the macaque study has been described in detail previously (10). Female squirrel monkeys (*Saimiri sciureus*) without preexisting immunity against MeV were obtained from the Experimental Biology Center of the INRS-Institut Armand-Frappier, and the experiments were approved by the Institutional Animal Care and Use Committee of the INRS-Institut Armand-Frappier. The animals were group housed in accordance with guidelines of the Canadian Council for Animal Care and the American Association for Accreditation of Laboratory Animal Care. Groups of six or eight animals were infected intranasally with $10^{4.5}$ $TCID_{50}$ of MeV-N4^{blind} or the wild-type MeV strain, respectively. The animals were monitored three times a week for clinical signs, including anorexia, depression, diarrhea, skin rash, and body temperature. Seven, 12, 14, and 21 days after infection, animals were anesthetized with ketamine (Pfizer) and weighed, and blood samples were collected. On days 7 and 12, three animals of each group, and the two remaining MeV-infected animals on day 21, were intubated to collect tracheal lavages and then sacrificed to harvest tissues for histological analyses and RNA isolation.

Virus quantification. To quantify the viral load in peripheral blood mononuclear cells (PBMCs), ammonium-chloride-potassium (ACK) lysis buffer (Invitrogen) was used to lyse erythrocytes and isolate white blood cells from EDTA-treated blood samples. The cells were counted and then cocultivated with Vero/hSLAM cells at serial 10-fold dilutions for 5 days. Virus titers were expressed as $TCID_{50}/10^6$ cells. For virus quantification in lymph node samples, tissue samples were weighed and a maximum of 500 mg was homogenized in 500 μl of DMEM with 10$\times$ penicillin-streptomycin (Invitrogen) for two rounds of 10 s at 6 m/s using a FastPrep-24 tissue homogenizer (MP Biomedicals), followed by a quick spin. Virus titers in lysate supernatant, tracheal lavage fluid, or urine were determined by limited dilution and expressed as $TCID_{50}$ per gram of tissue or milliliter of fluid, respectively.

Immune response assessment. For total white blood cell count, fresh heparinized blood was diluted 1:100 in water containing 3% acetic acid, and the remaining cells were counted. The remaining blood was used to isolate PBMCs by Ficoll purification for proliferation activity assessment. Toward this, purified PBMCs were resuspended in 100 μl RPMI (Invitrogen) with 10% FBS and activated with 1 μg/ml phytohemagglutinin (PHA; Invitrogen) or left untreated. After 24 h, bromodeoxyuridine (BrdU) was added, and the cells were incubated for another 24 h. The level of BrdU incorporation was quantified using the cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kit (Roche), and the proliferation index was expressed as the ratio of the average signal in stimulated and nonstimulated wells.

Immunostaining. Tissue samples were collected and fixed in 4% paraformaldehyde (Sigma) for at least 48 h. Fixed samples were either embedded in optimum cutting temperature (OCT) medium, flash-frozen in liquid nitrogen, and cut with a cryostat or paraffin embedded and cut with a microtome in 5-μm sections. Slides were left unstained or stained using monoclonal antibodies against MeV nucleoprotein (Millipore), in combination with reagents of the NovoLink Min polymer detection system (Leica), which contained the 3,3′-diaminobenzidine (DAB) chromogen and hematoxylin counterstain. The slides were mounted in Vectamount (Vector Laboratories).

***In vitro* infection of PBMCs.** PBMCs from human donors, pig-tailed macaques (*Macaca nemestrina*), and squirrel monkeys were purified by centrifugation through a Histopaque-1077 (Sigma) gradient. PBMCs were washed three times with phosphate-buffered saline (PBS) and then resuspended in 1 ml of RPMI 1640 containing 10% FBS and 1% L-glutamine (Sigma). PBMCs were seeded into round-bottom 96-well plates at a density of 2.5 $\times$ 10^5 cells per well, and 2 μg/ml PHA was added for activation. Cells were then infected with either MeV or MeV-N4[blind] at a multiplicity of infection (MOI) of 0.1 or left uninfected using a medium-only control. At different times after infection, samples were resuspended and fixed in 4% paraformaldehyde (PFA). GFP expression levels were quantified using an Accuri C6 flow cytometer (Becton Dickinson).

Statistical analyses. Prism (GraphPad) was used for all statistical analysis. Unpaired Student's *t* test was chosen to compare the two groups.

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